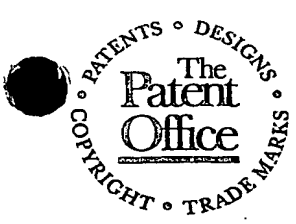

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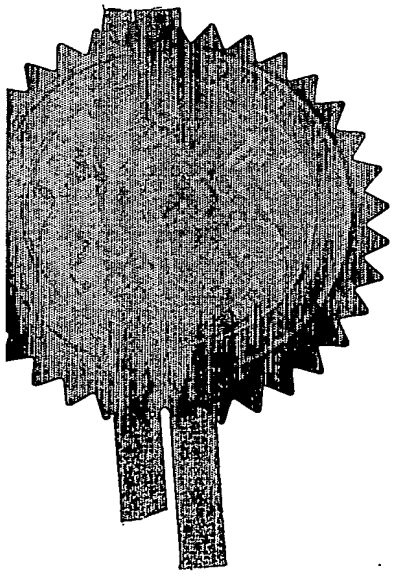

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1. Your reference — P.86130 JCI/SA

2. Patent application number *(The Patent Office will fill in this part)* — 0215710.5 — 05 JUL 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ISIS INNOVATION LIMITED
Ewert House, Ewert Place
Summertown, Oxford OX2 7SG
UNITED KINGDOM

Patents ADP number *(if you know it)* 39985640003

If the applicant is a corporate body, give the country/state of its incorporation — GB

4. Title of the invention — DIAGNOSTIC METHOD

5. Name of your agent *(if you have one)* — J.A. KEMP & CO.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

14 South Square
Gray's Inn
London
WC1R 5JJ

Patents ADP number *(if you know it)* 26001

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Number of earlier application	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature J.A. Kemp & Co. Date 5 July 2002

J.A. KEMP & CO.

12. Name and daytime telephone number of person to contact in the United Kingdom

IRVINE, Jonquil Claire
020 7405 3292

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DIAGNOSTIC METHOD

Field of the invention

The invention relates to a method of diagnosing the infection status of individuals.

Background of the invention

Infection by a pathogen may or may not cause disease symptoms in an individual. Although therapeutic products are available for treating infection by pathogens, prolonged use of these products may be harmful. Therefore it is desirable to target use of the therapeutic products to individuals who are more likely to develop disease symptoms. Targeting therapy in this way will also be more cost-effective.

Summary of the invention

Long term infected individuals who have not developed disease symptoms are much less likely to develop disease symptoms than recently exposed individuals. For example following exposure to M. tuberculosis individuals have a risk of approximately 10% of progressing to active tuberculosis with disease symptoms in the first one to two years following exposure. If active tuberculosis does not manifest within the first one to two years then the residual risk of progress to active tuberculosis is 5% over the remaining lifetime of the individual. It is therefore desirable to target recently infected individuals for preventative treatment because they have a high probability of progressing to disease.

Some groups of individuals have a higher risk for developing active tuberculosis, such as young children (less than 5 years old), newborn babies (less than 1 year old), individuals with HIV infection or on immunosuppressive medication such as corticosteroids (typically oral corticosteroids), such as prednisolone, or antibodies against TNF-α (typically monoclonal and/or humanised), such as infliximab. It is even more important to diagnose recent exposure to pathogen in such individuals.

The inventors have found that T cells from individuals recently exposed to an intracellular pathogen react to whole proteins from the pathogen but do not react to,

or show substantially less reaction to, peptide epitopes from the pathogen. It is believed that this may be because when a cellular T cell immune response has just been primed (induced) by a recent infection the T cells are of a lower affinity for their cognate ligand, as fine-tuning of the epitope specificity and clonal expansion of the different T cell populations is still going on. Since in T cell response assays whole protein is presented to T cells after uptake and processing by antigen presenting cells (APCs) followed by presentation of the optimal peptide epitopes in the context of MHC molecules at the APC surface, even cells of relatively low affinity will recognise these optimum naturally processed and presented epitopes.

In contrast short multiple peptide epitopes from the pathogen even when they together represent the entire sequence of a protein antigen are not normally the optimal epitopes, but merely tend to contain the optimum epitope sequence within their sequence. Therefore recognition of these peptides will require T cells to be present which are of higher affinity to the optimal epitope. Such T cells only appear in the later course of infection, when the T cell repertoire is more mature and focussed, and would not be present in recently exposed individuals.

Accordingly the invention provides a method of diagnosing in an individual recent exposure to an agent which is a pathogen, a vaccine or a moiety which causes a cellular immune response, said method comprising determining in vitro or in vivo whether the T cells of the individual recognise a protein from said agent having a length of at least 30 amino acids to a greater extent than a peptide epitope from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

Preferably in the method the pathogen is M. tuberculosis and the peptide epitope and/or protein is from ESAT-6 or CFP10.

Detailed description of the invention

The method of the invention comprises determining whether the T cells of an individual recognise a protein (with a length of at least 30 amino acids) from an agent to a greater extent than a peptide epitope from the agent. This may be done by detecting the reaction of the T cells either to the protein and peptide epitope or to an

analogue of either of these (discussed below). It is understood that herein reference to "protein" or "peptide epitope" from the pathogen will also include the analogues of these molecules unless the context requires otherwise.

The method is generally performed on a sample from an individual who is preferably a human, but may be an animal (typically an animal which can be naturally or artificially infected by the relevant pathogen). Thus the individual may be a mammal, such as a primate, cow, sheep, pig, badger or rodent, e.g. a mouse or rat. The individual may be at risk of (natural) exposure to the pathogen, for example the individual may live in an area in which the pathogen occurs. The individual may have an increased risk of becoming infected, typically for socio-economic reasons or may have a genetic or acquired predisposition to the pathogen. In one embodiment the exposure is not an artificial exposure, for example intentional exposure of an animal model to a pathogen. In another embodiment the exposure is to a non-natural (typically intentional) release of the pathogen in the area where the host (including humans) lives.

The pathogen may be an extracellular pathogen, but is preferably an intracellular pathogen, and is generally a naturally occurring pathogen (not modified artificially). The pathogen is typically able to infect any of the specific species of host mentioned herein. It may be a virus, bacterium or fungus, such as HPV, HIV, HCV, a Chlamydia species, HBV, EBV, CMV, HSV, Legionella, S. typhi, P. falciparum, Leishmaniasis, M. leprae, influenza virus, foot and mouth virus, a Toxoplasma species, a Brucella species, a Cryptococcus species, a Candida species or an Aspergillus species.

In a preferred embodiment the pathogen is a mycobacterium. The mycobacterium typically expresses ESAT-6 or CFP10, and may be *M.tuberculosis*. The mycobacterium may be *M.marinum* or *M.kansasii*. The pattern of clinical symptoms can be used to distinguish between these two organisms and *M.tuberculosis*. The mycobacterium may be *M. bovis* (which infects cows, but can also infect humans and other species such as badgers and monkeys).

In the case where the agent is a vaccine the vaccine may contain antigen from (provide protection against) any of the pathogens mentioned herein. Any of the types

of agent mentioned herein may be capable of inducing a cellular response in the individual, typically a T cell and/or NK cell response.

The protein and/or peptide epitope of the pathogen may be of any of the pathogens mentioned herein, preferably being mycobacterial. In one embodiment the protein is, or contains sequence from, a particular pathogen protein, whilst the peptide epitope is from a different protein from the pathogen. However preferably the peptide epitope is an epitope within the protein, i.e. the protein comprises the sequence of the peptide epitope. The peptide epitope generally contains within its sequence an optimal epitope, typically flanked by one or more amino acids at the N or C terminal end of the optimal epitope sequence.

The protein may be a membrane protein, a cytoplasmic protein (present in the cytoplasm of the pathogen or a cell which it has infected), a secreted protein (secreted from the pathogen and/or from the infected cell), an enzyme, a structural protein or a regulatory protein. The protein may be one which typically comprises at least 10%, such as at least 30% or 50% of the dry mass of the agent. The protein may be one which in its natural form and/or as used in the method comprises at least 5, such as at least 10 or 15 CD4 and/or CD8 T cell epitopes.

The peptide epitope is typically a fragment of any of the pathogen proteins mentioned. In the case of M. tuberculosis the peptide epitope may be any of the peptides shown below from ESAT-6 and CFP-10.

Peptides from ESAT-6:

MTEQQWNFAGIEAAA
WNFAGIEAAASAIQG
IEAAASAIQGNVTSI
SAIQGNVTSIHSLLD
NVTSIHSLLDEGKQS
HSLLDEGKQSLTKLA
EGKQSLTKLAAAWGG
LTKLAAAWGGSGSEA

AAWGGSGSEAYQGVQ
SGSEAYQGVQQKWDA
YQGVQQKWDATATEL
QKWDATATELNNALQ
TATELNNALQNLART
NNALQNLARTISEAG
NLARTISEAGQAMAS
ISEAGQAMASTE GNV
QAMASTEGNVTGMFA

Peptides from CFP-10:

MAEMKTDAATLAQEA
TDAATLAQEAGNFER
LAQEAGNFERISGDL
GNFERISGDLKTQID
ISGDLKTQIDQVEST
KTQIDQVESTAGSLQ
QVESTAGSLQGQWRG
AGSLQGQWRGAAGTA
GQWRGAAGTAAQAAV
AAGTAAQAAVVRFQE
AQAAVVRFQEAANKQ
VRFQEAANKQKQELD
AANKQKQELDEISTN
KQELDEISTNIRQAG
EISTNIRQAGVQYSR
IRQAGVQYSRADEEQ
VQYSRADEEQQQALS
ADEEQQQALSSQMGF

The peptide epitope typically has a length of at least 8 to 29 amino acids, such as 12 to 25 amino acids. The protein typically has a length of at least 30 to 400 amino acids, such as 50 to 300, or 80 to 200 amino acids in length. The protein may be the same as the whole naturally occurring protein, or a fragment thereof. In one embodiment it is in the form of a fusion protein, for example with non-pathogen protein sequence. Generally the protein comprises a pathogen sequence (sequence from a protein of the pathogen) which is at least 8, for example at least 12, 18, 25 or 30 amino acids long.

The method of the invention is generally based on the detection of different levels of response from and/or different frequencies of T cells in an individual to one or more proteins and one or more (smaller) peptide epitopes from a pathogen. The T cells which react are specific for/bind to amino acid sequence in the protein or peptide epitope. The T cells which are analysed in the method may be CD4 and/or CD8 T cells, γδ T cells or CD1 restricted T cells. The T cells have been pre-sensitised *in vivo* to protein from the pathogen.

The method of the invention may be performed using any suitable technique, for example a technique which detects T cell reaction to a protein/peptide epitope. In many such techniques whether or not the T cells of the individual react to the protein or peptide epitope will be readily apparent, and thus individuals will be diagnosed as having been recently exposed if their T cells react to the protein and do not react to the peptide epitope. Arbitrary thresholds may be used to determine positive and negative responses.

Typically the method will be performed in a manner in which reactive T cells present at a frequency of at least about 20 per million peripheral blood mononuclear cells (PBMCs) will be detectable (a positive result), and preferably distinguishable from a reactive T cells present at a frequency of about 19 per million PBMCs or less (a negative result).

Thus individuals will typically be selected as being exposed recently to pathogen if they are found to have T cells which are able to recognise the protein at a frequency of at least 20 per million PBMCs and if they are found to have less than 19 per million PBMCs which recognise the peptide epitope. It is understood though

that a positive and negative result may be defined using thresholds different from these specific thresholds.

In a preferred embodiment the T cells are detected by:

(i) contacting in vitro or in vivo a first population of T cells from the individual with one or more peptide epitopes from the pathogen (including an analogue of said peptide which is recognised by T cells that recognise said peptide), and determining the reaction of the T cells to the peptide epitope(s), and

(ii) contacting in vitro or in vivo a second population of T cells from the individual with a protein from the pathogen (including an analogue of said protein which is recognised by T cells that recognise said protein), wherein the protein has a length of at least 30 amino acids and determining the reaction of the T cells to the protein.

Determination of whether the T cells react to/recognise the protein or peptide epitope is typically done by detecting a change in the state of the T cells in the presence of the protein or peptide epitope. The change in state is generally caused by antigen specific functional activity of the T cell after the T cell receptor binds the protein (after it is processed) or peptide epitope. Generally when binding the T cell receptor the processed protein or peptide is bound to an MHC class I or II molecule, which is typically present on the surface of an antigen presenting cell (APC).

The change in state of the T cell may be the start of or increase in secretion of a substance from the T cell, such as a cytokine, especially IFN-γ, IL-2 or TNF-α. Determination of IFN-γ secretion is particularly preferred. Intracellular changes may be detected, for example by using intracellular staining techniques, typically intracellular cytokine staining (e.g. for any of the cytokines mentioned herein). The staining can be detected using a cell sorting technique, for example using a FACS technique.

The substance can typically be detected by allowing it to bind to a specific binding agent and then measuring the presence of the specific binding agent/substance complex. The specific binding agent is typically an antibody, such as polyclonal or monoclonal antibodies. Antibodies to cytokines are commercially available, or can be made using standard techniques.

Typically the specific binding agent is immobilised on a solid support. In one embodiment this allows the actual number of responding T cells to be determined since after binding the agent the substance will remain in the vicinity of the T cell which secreted it. Thus 'spots' of substance/agent complex may form on the support, each spot representing a T cell which is secreting the substance. Quantifying the spots (and typically comparing against a control) allows determination of recognition of the peptide.

After the substance is allowed to bind, the solid support can optionally be washed to remove material which is not specifically bound to the agent. The agent/substance complex may be detected by using a second binding agent which will bind the complex. Typically the second agent binds the substance at a site which is different from the site which binds the first agent. The second agent is preferably an antibody and is labelled directly or indirectly by a detectable label.

Thus the second agent may be detected by a third agent which is typically labelled directly or indirectly by a detectable label. For example the second agent may comprise a biotin moiety, allowing detection by a third agent which comprises a streptavidin moiety and typically alkaline phosphatase as a detectable label.

In one embodiment the detection system which is used is the *ex-vivo* ELISPOT assay described in WO 98/23960. In that assay IFN-γ secreted from the T cell is bound by a first IFN-γ specific antibody which is immobilised on a solid support. The bound IFN-γ is then detected using a second IFN-γ specific antibody which is labelled with a detectable label. Other detectable labels may be used.

Typically the T cells used in the method are taken from the individual in a blood sample, although other types of body sample which contain T cells can be used. The sample may be added directly to the assay or may be processed first. Typically the processing may comprise diluting of the sample, for example with water or buffer. Typically the sample is diluted from 1.5 to 100 fold, for example 2 to 50 or 5 to 10 fold.

The processing may comprise separation of components of the sample. Typically mononuclear cells (MCs) are separated from the samples. The MCs will comprise the T cells and APCs. Thus in the method the APCs present in the

separated MCs can present peptide to the T cells. In another embodiment only T cells, such as only CD4 T cells, can be purified from the sample. PBMCs, MCs and T cells can be separated from the sample using techniques known in the art, such as those described in Lalvani *et al* (1997) *J.Exp. Med.* **186**, p859-865.

In one embodiment the T cells which are detected are in the form of unprocessed or diluted samples. The T cells are preferably directly *ex vivo*, i.e. they are not cultured before being used in the method. The T cells are typically freshly isolated T cells (such as in the form of freshly isolated MCs or PBMCs).

The APC which is typically present in the method may be from the same individual as the T cell or from a different individual. The APC may be a naturally occurring APC or an artificial APC. The APC is a cell which is capable of presenting peptide to a T cell. It is typically a B cell, dendritic cell or macrophage. It is typically separated from the same sample as the T cell and is typically co-purified with the T cell. Thus the APC may be present in MCs or PBMCs. The APC is typically a freshly isolated *ex vivo* cell or a cultured cell. It may be in the form of a cell line, such as a short term or immortalised cell line. The APC may express empty MHC class I or II molecules on its surface.

In one embodiment of the method more than one protein from the pathogen (typically at least 2, 5, 10 or more different proteins) and/or more than one peptide epitope from the pathogen (typically at least 2, 5, 10 or more different peptide epitopes) may be used. Thus, for example, the T cells can be placed into an assay with all the proteins or peptide epitopes (i.e. a pool of the proteins or peptides) which it is intended to test. Alternatively the T cells can be divided and placed into separate assays each of which contain one or some of the proteins or peptides which it is intended to test.

In one embodiment the protein or peptide epitope is provided to the APC in the absence of the T cell. The APC is then provided to the T cell, typically after being allowed to present the processed protein or peptide epitope on its surface. Presented peptide may have been taken up inside the APC and presented, or simply be taken up onto the surface without entering inside the APC.

The duration for which the protein or peptide epitope is contacted with the T

cells will vary depending on the method used for determining recognition. Typically 10^4 to 10^7, preferably $1x10^5$ to $5x10^5$ PBMCs are added to each assay. The peptide is typically used in the assay at a concentration of from 10^{-1} to 10^3μg/ml, preferably 0.5 to 50μg/ml or 1 to 10μg/ml.

Typically the length of time for which the T cells are incubated with the protein or peptide is from 4 to 72 hours, preferably 6 to 48, 8 to 24 or 10 to 16 hours. When using *ex vivo* PBMCs it has been found that $0.3x10^6$ PBMCs can be incubated in 10μg/ml of peptide for 12 hours at 37°C.

The method may be based on an ELISA method, such as the whole blood Quantiferon system (for example as available from Cellestis).

In one embodiment instead of the protein and/or peptide epitope analogues are used which are recognised by T cells which recognise the protein or peptide. Thus such analogues may be identified by routine means and their ability to be recognised by the relevant T cells can be tested using any suitable technique mentioned herein. For the proteins such recognition will of course be after processing and presentation of the protein and/or analogue by an APC.

The analogue will generally have similar binding properties to the protein and/or peptide and thus typically binds to the same MHC molecule. The analogue may bind to antibodies specific for the protein or peptide, and thus may inhibit binding of the protein or peptide to such an antibody.

The analogue is typically a protein or peptide. It may have homology with the equivalent original protein or peptide. A peptide which is homologous to another peptide is typically at least 70% homologous to the peptide, preferably at least 80 or 90% and more preferably at least 95%, 97% or 99% homologous thereto, for example over a region of at least 8, preferably at least 15, for instance at least 40, 60 or 100 or more contiguous amino acids. The analogue typically differs from the protein or peptide by 1, 2, less than 6, such as less than 12 mutations (each of which is a substitution (e.g. a conservative substitution), deletion or insertion) for example over any of the above-mentioned lengths of region mentioned for homology.

Methods of measuring protein homology are well known in the art and it will be understood by those of skill in the art that in the present context, homology is

calculated on the basis of amino acid identity (sometimes referred to as "hard homology"). For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395).

An analogue which is a protein or peptide typically has any of the amino acid lengths mentioned above for the protein or peptide discussed above and/or may be part of a fusion protein. Typically the amino acids in the analogue at the equivalent positions to amino acids in the original protein or peptide which contribute to binding the MHC molecule or are responsible for the recognition by the T cell receptor, are the same or are conservative changes.

Conservative substitutions are defined in the table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Typically the analogue of the protein or peptide comprises one or more modifications, which may be natural post-translation modifications or artificial modifications. The modification may provide a chemical moiety (typically by substitution of a hydrogen, e.g. of a C-H bond), such as an amino, acetyl, hydroxy or halogen (e.g. fluorine) group or carbohydrate group. Typically the modification is present on the N or C terminus.

The analogues may comprise one or more non-natural amino acids, for example amino acids with a side chain different from natural amino acids.

Generally, the non-natural amino acid will have an N terminus and/or a C terminus. The non-natural amino acid may be an L- or D- amino acid. The analogues typically has a shape, size, flexibility or electronic configuration which is substantially similar to the original protein or peptide. It is typically a derivative of the original protein or peptide.

The analogue is typically designed by computational means and then synthesised using methods known in the art. Alternatively the analogue can be selected from a library of compounds. The library may be a combinatorial library or a display library, such as a phage display library. The library of compounds may be expressed in the display library in the form of being bound to a MHC class I or II molecule, such as the MHC molecule which the original peptide binds. Analogues are generally selected from the library based on their ability to mimic the binding characteristics of the original protein or peptide. Thus they may be selected based on ability to bind a T cell receptor or antibody which recognises the original protein or peptide.

In one embodiment the T cells are detected not based on their response to a substance but based on their ability to bind a specific binding agent. Typically the agent is or comprises any of the proteins, peptide epitopes or analogues mentioned herein. The agent may be labelled (for example using any of the detectable labels mentioned herein). The specific binding agent may comprise an MHC molecule, and is preferably an MHC tetramer-peptide complex.

The peptide or analogue discussed herein can be made using standard synthetic chemistry techniques, such as by use of an automated synthesizer. They can be made from a longer polypeptide, e.g. a fusion protein, which polypeptide typically comprises the sequence of the peptide, and may be derived from the polypeptide by for example hydrolysing the polypeptide, such as using a protease; or by physically breaking the polypeptide. The protein may be expressed recombinantly.

In the case here the method is performed in vivo the protein, peptide epitope and/or analogue may be administered by any suitable means and at any suitable dose, for example in the form, by the route or at the dosage discussed for the therapeutic

product below. Administration to the skin is preferred.

The invention also provides a method of treating an individual comprising administering to an individual diagnosed as having been recently exposed to a pathogen by the diagnosis method a product which prevent or treats the condition caused by the pathogen. Thus the invention provides use of the product in the manufacture of a medicament for the treatment of an individual who has been diagnosed as having been recently exposed to the pathogen by a method of the invention. Typically a non-toxic effective amount of the therapeutic agent is administered.

In the case M. tuberculosis the therapeutic agent may be rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicyclic acid, kanamyin, capreomycin, ethionamide, cycloserine, thiacetazone or a flouroquinolone (e.g. ciprofloxacin).

The product may be in the form of a pharmaceutical composition which comprises the agent and a pharmaceutically acceptable carrier or diluent. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. Typically the product is administered by parenteral, intravenous, intramuscular, subcutaneous, transdermal, intradermal, oral, intranasal, inhalation (into the lungs), intravaginal, or intrarectal administration.

The dose of the product may be determined according to various parameters, especially according to the particular agent; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular patient. A suitable dose may however be from 10μg to 10g, for example from 100 μg to 1g of the product.

The inventors have also shown using a T cell detection (ex vivo ELISPOT) based technique that T cells from the individuals exposed to a pathogen reacted to antigen from the pathogen at 3 months from exposure, but no longer reacted to antigen at 6 months from exposure. Given that the presence of effector T cells indicates the presence of infection by a pathogen this shows these individuals had cleared the infection which had been detected initially. This elucidation of the the

dynamics of the T cell response during infection and clearance of infection shows the need to test at a subsequent time point to avoid treating individuals who naturally clear infection.

Accordingly the invention provides a method of diagnosing an individual who has cleared an infection by a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the individual has cleared the infection.

The infection may be cleared naturally by the immune response of the individual but could also be cleared pharmacologically by use of a product which treats the infection.

In addition the inventors have identified individuals whose T cells did not react at 3 months from exposure but did react at 6 months from exposure. These individuals are mounting a slower weaker response to infection. They are therefore less likely to be able to control the infection and are more likely to progress to active disease. It is therefore desirable to target this group of individuals for treatment.

Accordingly the invention provides a method of diagnosing an individual who is more likely to progress to active disease after exposure to a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and subsequent second time point after exposure to the pathogen, wherein the finding that the T cells do not recognise the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is more likely to progress to active disease.

The finding may also be used in a method of diagnosing an individual who mounts a weaker response to a vaccine or a moiety which induces a cellular response after exposure to the vaccine or moiety comprising determining whether the T cells of the individual recognise antigen from the vaccine or moiety at a first and subsequent second time point after exposure, wherein the finding that the T cells do not recognise the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is mounting a weaker response to the

vaccine or moiety.

Further testing at a subsequent time point may also be used to test the efficacy of a vaccine. Accordingly the invention provides a method of testing the efficacy of a vaccine which has been administered to an individual comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the vaccine antigen has been cleared and is not persisting.

If the vaccine is found to have low efficacy (i.e. is being cleared) then the individual may need to be vaccinated with an alternative vaccine.

In the above methods the first time point and second time point are typically separated by about 2 to 16 weeks, such as about 4 to 12 weeks. The first time point may be about 8 to 16 weeks (preferably about 12 weeks) after exposure and/or the second time point may be about 18 to 48 weeks (preferably about 24 weeks) after exposure.

The individuals who are diagnosed may be any of the individuals who are mentioned herein. The said determining of T cell recognition may be carried out using any suitable method, such as any suitable method disclosed herein. The antigen may be any antigen which is recognised by T cells (such as any type of T cell mentioned herein) and thus may be any of the proteins or peptide epitopes mentioned herein. Analogues of the antigen may be used in the determination such as any of the types of, or specific, analogues mentioned herein

Typically the pathogen is an intracellular pathogen such as any such pathogen mentioned herein, for example HPV, HIV, SIV, HCV, a Chlamydia species, HBV, EBV, CMV, HSV, Legionella, S. typhi, P. falciparum, Leishmaniasis, M. leprae, influenza virus, foot and mouth virus, a Toxoplasma species, a Brucella species, a Cryptococcus species, a Candida species or an Aspergillus species. Preferably the pathogen is M. tuberculosis.

As mentioned above the recognition of the antigen or analogue may be determined using any suitable method, but is preferably determined by detecting secretion of a cytokine from the T cells, such as IFN-γ.

The above method which involves determining T cell recognition at two time points may be used to diagnose individuals who have a high probability of progressing to active disease. The invention provides a method of treating an individual who has been diagnosed in this way comprising administering to the individual a product which prevent or treats the condition caused by the pathogen.

Preferably the pathogen is M. tuberculosis and/or the agent is rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicyclic acid, kanamyin, capreomycin, ethionamide, cycloserine, thiacetazone or a flouroquinolone, or an analogue of such an agent.

The inventors have realised that detection of latent mycobacterial infection, and therefore of susceptibility to active tuberculosis, in individuals on immunosuppressive therapy may be determined using the T cell based assays described herein.

Accordingly the invention provides a method of diagnosing susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on or about to start immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis disease and latent mycobacterial infection.

In addition the invention provides a method of monitoring susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis disease and latent mycobacterial infection.

The immunosuppressive therapy in these methods may comprise administration of an anti-TNF-α antibody, such as infliximab. The individuals who are diagnosed or monitored may be any of the types of individuals who are mentioned herein. The said determining of T cell recognition may be carried out using any suitable method, such as any suitable method disclosed herein. The mycobacterium may be any such mycobacterium mentioned herein, but is preferably

M. tuberculosis. The antigen may be any antigen which is recognised by T cells (such as any type of T cell mentioned herein) and thus may be any of the proteins or peptide epitopes mentioned herein. Analogues of the antigen may be used in the determination such as any of the types of, or specific, analogues mentioned herein

The invention is illustrated by the following Examples:

Example 1

Methods

Ex vivo ELISPOT assays

ELISPOT assays were performed 2-4 hr after venepuncture. Samples were processed and scored by two scientists without reference to personal identifiers or TST results. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by standard density centrifugation and washed in RPMI. PBMC were counted in an automated cell counter under a microscope, resuspended in complete medium (R10), and plated at 2.5 x 10^5 cells per well in ELISPOT plates pre-coated with catcher anti-IFN-γ monoclonal antibody (mAb) (Mabtech, Stockholm, Sweden) and pre-blocked with R10.

Duplicate wells contained no antigen (negative control), phytohaemagglutinin (positive control) (ICN Biomedicals, OH, USA), recombinant ESAT-6 (rESAT-6) or one of 12 different peptide pools derived from ESAT-6 and CFP10. Assays were incubated overnight at 37C, 5% CO_2, and developed the next morning by washing the plates with phosphate buffered saline 0.05% Tween-20 (Sigma, MO, USA), incubating for 90 min with detector anti-IFN-γ mAb preconjugated to alkaline phosphatase (Mabtech), repeat washing and 15 min incubation with BCIP/NBT^{PLUS} chromogenic substrate (Moss Inc, MD, USA). Plates were air dried after washing in tap water.

Assays were scored in an automated ELISPOT counter with the same settings for all samples. Test wells were scored as positive if they contained a mean of at least 5 spot forming cells (SFCs) more than the mean of the negative control wells, and, in addition, this number was at least twice the mean of the negative control wells. For peptide pools, a positive was defined as response to pools in both arrays as each array contained a full set of peptides. A positive response to pools of ESAT-6-derived

peptides, pools of CFP10-derived peptides or rESAT-6 was deemed a positive ELISPOT assay.

Peptides

As previously described, 17 peptides spanning the length of the ESAT-6 molecule and 18 peptides spanning the length of the CFP10 molecule were purchased (Research Genetics, AL, USA). Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues; purity was >70%. Peptides were arranged into 12 pools comprising 2 arrays of 6 pools each where each array contained all 35 peptides from the two molecules in contrasting combinations, so that each peptide was tested in quadruplicate.

Results

124 individuals from an Italian hospital with recent (11 weeks previously) exposure to (and therefore risk of infection with) M. tuberculosis were tested using the ELISPOT assay described above. Using the pools of peptides and the whole antigens for both ESAT-6 and CFP-10 only 10 (8%) were found to be positive by tuberculin skin test whereas 34 (27%) were found to be positive using ELISPOT showing that ELISPOT detects infection earlier after exposure. Of the 124 individuals, 35 were health care workers (HCWs), several of whom may have have been previously exposed to M. tuberculosis in the distant past, and 89 were mothers and their 11 week old babies, of whom no babies and only very few mothers might have been previously exposed. Of the 18 HCWs who responded to the ELISPOT 11 (61%) responded to whole antigen only. Of the 16 mothers and babies who responded 14 (87.5%) responded responded to whole antigen to a greater extent than than to the pools of peptides. In this case the 16 mothers and babies responded only to whole antigen and did not respond to the pools of peptides.

In contrast in a study of 545 children in a M. tuberculosis outbreak at a UK secondary school, where children were exposed to M. tuberculosis 4 to 12 months prior to testing by ELISPOT, 133 children responded to whole ESAT-6 or peptides from ESAT-6, and from these only 13 (9.8%) responded to whole ESAT-6 only

(compared with the above figures of 61% and 87.5% in the Italian study). This reflects the fact that the children in the UK school were exposed much earlier than the individuals in the Italian study. Therefore the detection of a higher response to whole antigen than to peptides may be used to detect recent exposure to a pathogen.

Example 2

Results of two stage testing (two test ELISPOT format) at 3 and 6 months after M. tuberculosis exposure

A T cell recognition based assay (ex vivo ELISPOT) was used to study the dynamics of early infection at the T cell level (in contrast to symptom based infection/disease indicators). The detectable presence in an individual of effector T cells is believed to indicate that the host is currently infected by a pathogen.

The donors were 108 mothers and babies from the Italian hospital study described above. It was found that 61 donors who had tested negative in the ELISPOT assay at 3 months tested negative again at 6 months. In addition 15 donors who had tested positive at 3 months also tested positive at 6 months. However 13 donors who had tested negative at 3 months tested positive at 6 months, and 19 donors who had tested positive at 3 months then tested negative at 6 months. Therefore by the use of assaying T cells of individuals at two time points after exposure two important groups of individuals were identified.

Firstly the 19 donors who tested positive at the first time point and negative at the second time point are individuals initially became infected and then cleared the infection. These individuals will not require treatment. This illustrates the importance of testing a subsequent second time after an initial positive testing when using T cell based diagnosis of infection.

Secondly the 13 donors who tested negative at the first time point and positive at the second time point are individuals who have mounted a slower (weaker) T cell response. Such individuals with weaker responses are more likely to fail to control infection, and hence to develop active disease. Thus a T cell based two time point diagnostic test can be used to identify individuals who are more likely to progress to active disease. Clearly it is desirable to target these individuals for

therapy. This finding may also be used to identify individuals who mount a slower response to vaccines or to other moieties which induce a cellular response.

Sequence of ESAT-6:

MTEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTK
LAAAWGGSGSEAYQGVQQKWDATATELNNALQNLAR
TISEAGQAMASTE GNVTGMFA

Sequence of CFP-10:

MAEMKTDAATLAQEAGNFERISGDLKTQIDQVESTAGS
LQGQWRGAAGTAAQAAVVRFQEAANKQKQELDEIST
NIRQAGVQYSRADEEQQQAL S SQMGF

CLAIMS

1. Method of diagnosing in an individual recent exposure to an agent which is a pathogen, vaccine or any other moiety which induces a cellular response, said method comprising determining in vitro whether the T cells of the individual recognise a protein from said agent having a length of at least 30 amino acids, to a greater extent than one or more peptide epitopes from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

2. Method according to claim 1 comprising determining whether T cells of the individual exhibit a greater reaction to a protein from said agent having a length of at least 30 amino acids than to one or more peptide epitopes from the agent, a greater reaction indicating that the individual has recently been exposed to the agent.

3. Method according to claim 1 wherein determining whether the T cells recognise said protein is performed by determining the reaction of the T cells to an analogue of the protein which is recognised by T cells which recognise said protein, wherein said analogue has a length of at least 30 amino acids.

4. Method according to claim 1 or 3 wherein determining whether the T cells recognise said peptide epitope is performed by determining the reaction of the T cells to an analogue of the peptide epitope which analogue is recognised by T cells which recognise said peptide epitope.

5. Method according to any one of the preceding claims comprising:
(i) contacting a first population of T cells from the individual with (a) one or more peptide epitopes from the agent, or (b) an analogue of said peptide(s) which is recognised by T cells that recognise said peptide(s), and determining the reaction of the T cells to the peptide(s) or analogue(s), and
(ii) contacting a second population of T cells from the individual with (a) a protein from the agent, or (b) an analogue of said protein which is recognised by T cells that

recognise said protein, wherein the protein or analogue has a length of at least 30 amino acids and determining the reaction of the T cells to the protein or analogue.

6. Method according to any one of the preceding claims in which the individual is diagnosed as having been exposed to the agent recently if there is substantially no reaction of the T cells to the peptide epitope or an analogue thereof.

7. Method according to any one of the preceding claims in which protein or its analogue comprises at least the amino acid sequence of the peptide epitope or its analogue.

8. Method according to any one of the preceding claims in which the peptide epitope, or the analogue of the peptide epitope, has a length of 8 to 29 amino acids.

9. Method according to any one of the preceding claims wherein whether or not the T cells recognise a pool of a least 4 peptide epitopes, or analogues thereof, is determined.

10. Method according to any one of the preceding claims wherein a pool of peptide epitopes and/or analogues which together represent all of the possible epitopes from the protein is used.

11. Method according to any one of the preceding claims in which during detection of the reaction of the T cells to the protein, or the analogue of the protein, antigen presenting cells are present which are capable of processing the protein and presenting it to the T cells.

12. Method according to any one of the preceding claims wherein the pathogen is an intracellular pathogen or the vaccine is against an intracellular pathogen.

13. Method according to any one of the preceding claims wherein the pathogen is

HPV, HIV, SIV, HCV, a Chlamydia species, HBV, EBV, CMV, HSV, Legionella, S. typhi, P. falciparum, Leishmaniasis, M. leprae, influenza virus, foot and mouth virus, a Toxoplasma species, a Brucella species, a Cryptococcus species, a Candida species or an Aspergillus species; or the vaccine is against any of these pathogens.

14. Method according to any one of claims 1 to 12 wherein the pathogen is M. tuberculosis or the vaccine is against M. tuberculosis.

15. Method according to claim 14 wherein the protein and/or epitope peptide is from ESAT-6 or CFP10.

16. Method according to any one of the preceding claims wherein the peptide(s) is chosen from one or more of the following peptide epitopes:

MTEQQWNFAGIEAAA
WNFAGIEAAAASAIQG
IEAAAASAIQGNVTSI
SAIQGNVTSIHSLLD
NVTSIHSLLDEGKQS
HSLLDEGKQSLTKLA
EGKQSLTKLAAAWGG
LTKLAAAWGGSGSEA
AAWGGSGSEAYQGVQ
SGSEAYQGVQQKWDA
YQGVQQKWDATATEL
QKWDATATELNNALQ
TATELNNALQNLART
NNALQNLARTISEAG
NLARTISEAGQAMAS
ISEAGQAMASTEGNV
QAMASTEGNVTGMFA
MAEMKTDAATLAQEA

TDAATLAQEAGNFER
LAQEAGNFERISGDL
GNFERISGDLKTQID
ISGDLKTQIDQVEST
KTQIDQVESTAGSLQ
QVESTAGSLQGQWRG
AGSLQGQWRGAAGTA
GQWRGAAGTAAQAAV
AAGTAAQAAVVRFQE
AQAAVVRFQEAANKQ
VRFQEAANKQKQELD
AANKQKQELDEISTN
KQELDEISTNIRQAG
EISTNIRQAGVQYSR
IRQAGVQYSRADEEQ
VQYSRADEEQQQALS
ADEEQQQALSSQMGF

or an analogue thereof which is recognised by a T cell which recognises the peptide epitope.

17. Method according to any one of the preceding claims wherein recognition of the peptide epitope, or its analogue, or of the protein, or its analogue, is determined by detecting secretion of a cytokine from the T cells.

18. Method according to claim 17 in which the cytokine is IFN-γ.

19. Method according to claim 17 or 18 in which the cytokine is detected by allowing the cytokine to bind to an immobilised antibody specific to the cytokine and then detecting the presence of the antibody/cytokine complex.

20. Method of diagnosing in an individual recent exposure to an agent which is

pathogen, vaccine or any other moiety which induces a cellular response, said method comprising determining in vivo whether the T cells of the individual recognise a protein from said agent having a length of at least 30 amino acids, to a greater extent than a peptide epitope from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

21. Method according to claim 20 which is performed using the method of any one of claims 2 to 19.

22. Use a protein having a length of at least 30 amino acids from an agent which is a pathogen, vaccine or any other moiety which induces a cellular response and/or one or more peptide epitopes from the agent in the manufacture of a diagnostic means for use in a method of diagnosing in an individual recent exposure to the agent, said method comprising determining whether the T cells of the individual recognise the protein to a greater extent than peptide epitope(s), a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

23. A product comprising a protein from an agent which is a pathogen, vaccine or any other moiety which induces a cellular response, said protein having a length of at least 30 amino acids, and/or one or more peptide epitopes from the agent for separate, simultaneous or sequential use in a method of diagnosing in an individual recent exposure to the agent, said method comprising determining whether the T cells of the individual recognise the protein to a greater extent than the peptide epitope(s), a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

24. Method of treating an individual comprising administering to an individual diagnosed as having been exposed recently to a pathogen by a method according to any of the preceding claims, a product which prevent or treats the condition caused by the pathogen.

25. Use of a product which prevents or treats a condition caused by a pathogen in the manufacture of a medicament for the therapy of an individual who has been diagnosed as having been recently exposed to the pathogen by a method according to any one of claims 1 to 21.

26. Method or use according to claim 24 or 25 wherein the pathogen is M. tuberculosis and/or the agent is rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicyclic acid, kanamyin, capreomycin, ethionamide, cycloserine, thiacetazone or a flouroquinolone, or an analogue of such an agent.

27. A kit for carrying out the method of any one of claims 1 to 21 comprising (i) said epitope peptide or said analogue thereof, and (ii) said protein or said analogue thereof, and optionally also a means to detect whether T cells recognise (i) and (ii).

28. A kit according to claim 27 which also comprises an agent as defined in any one of claims 24 to 26.

29. Method of diagnosing an individual who has cleared an infection by a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the individual has cleared the infection.

30. Method of diagnosing an individual who is more likely to progress to active disease after exposure to a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and subsequent second time point after exposure to the pathogen, wherein the finding that the T cells do not recognise the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is more likely to progress to active disease.

31. Method of diagnosing an individual who mounts a weaker response to a vaccine or a moiety which induces a cellular response after exposure to the vaccine or moiety comprising determining whether the T cells of the individual recognise antigen from the vaccine or moiety at a first and subsequent second time point after exposure, wherein the finding that the T cells do not recognise the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is mounting a weaker response to the vaccine or moiety.

32. Method of testing the efficacy of a vaccine which has been administered to an individual comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the vaccine antigen has been cleared and is not persisting.

33. Method according to any one of claims 29 to 32 wherein the first time point and second time point are separated by about 12 weeks.

34. Method according to any one of claims 29 to 33 wherein the first time point is about 2 to 16 weeks after exposure and/or the second time point is about 18 to 48 weeks after exposure.

35. Method according to any one of claims 29 to 34 wherein said determining is performed by detecting whether the T cells of the individual recognise/react to an antigen from said pathogen, vaccine or moiety.

36. Method according to any one of claims 29 to 35 wherein the antigen is a protein from said pathogen, vaccine or moiety having a length of at least 30 amino acids or is a peptide epitope from the pathogen, vaccine or moiety, wherein the protein or peptide epitope are as defined in any one of the preceding claims.

37. Method according to any one of claims 29 to 36 wherein said determining is performed by detecting the reaction of the T cells to an analogue of antigen which is optionally an analogue as defined in any one of the preceding claims.

38. Method according to any one of claims 29 to 37 wherein said determining is performed by contacting in vitro or in vivo a population of T cells from the individual with the antigen, or an analogue of said antigen and determining the reaction of the T cells to the antigen or analogue.

39. Method according to any one of claims 29 to 38 wherein the determination is deemed negative if there is substantially no reaction of the T cells to the antigen or analogue.

40. Method according to any one of the claims 29 to 39 wherein said determining comprises detecting whether or not the T cells recognise a pool of a least 4 peptide epitopes, or analogues thereof.

41. Method according to any one of claims 29 to 40 wherein during said determining antigen presenting cells are present which are capable of processing the antigen or analogue and presenting it to the T cells.

42. Method according to any one of claims 29 to 41 wherein the pathogen is an intracellular pathogen or the vaccine is against an intracellular pathogen.

43. Method according to any one of claims 29 to 42 wherein the pathogen is HPV, HIV, SIV, HCV, a Chlamydia species, HBV, EBV, CMV, HSV, Legionella, S. typhi, P. falciparum, Leishmaniasis, M. leprae, influenza virus, foot and mouth virus, a Toxoplasma species, a Brucella species, a Cryptococcus species, a Candida species or an Aspergillus species; or the vaccine is against any of these pathogens.

44. Method according to any one of claims 29 to 42 wherein the pathogen is M.

tuberculosis or the vaccine is against M. tuberculosis.

45. Method according to any one of claims 29 to 44 wherein recognition of the antigen or analogue is determined by detecting secretion of a cytokine from the T cells.

46. Method according to claim 45 in which the cytokine is IFN-γ.

47. Method according to claim 45 or 46 in which the cytokine is detected by allowing the cytokine to bind to an immobilised antibody specific to the cytokine and then detecting the presence of the antibody/cytokine complex.

48. Method of treating an individual comprising administering to an individual diagnosed as being infected by a pathogen by a method according to any of claims 30 to 47, a product which prevent or treats the condition caused by the pathogen.

49. Method according to claim 48 wherein the pathogen is M. tuberculosis and/or the agent is rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicyclic acid, kanamyin, capreomycin, ethionamide, cycloserine, thiacetazone or a flouroquinolone, or an analogue of such an agent.

50. Use of an antigen or analogue as defined in any one of claims 29 to 47 in the manufacture of a diagnostic means for use in a method according to any one of claims 29 to 47 wherein the method is performed in vivo.

51. A kit for carrying out the method of any one of claims 29 to 47 comprising the antigen or analogue, and optionally also a means to detect whether T cells recognise antigen or analogue.

52. A kit according to claim 51 which also comprises an agent as defined in claim 48 or 49.

53. Method of diagnosing susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on or about to start immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis disease and latent mycobacterial infection.

54. Method of monitoring susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis disease and latent mycobacterial infection.

55. Method according to claim 53 or 54 wherein the immunosuppressive therapy comprises administration of an anti-TNF-α antibody.

56. Method according to claim 54 wherein the antibody is infliximab.

57. Method according to any one of claims 53 to 56 wherein T cell recognition is performed using a method as defined in any one of the preceding claims.